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(54) **Process for producing r(-)-mandelic acid and derivative thereof.**

(57) A process for treating R,S-mandelonitrile or a derivative thereof or a mixture of benzaldehyde with hydrogen cyanide with a microorganism belonging to the genus Rhodococcus in an almost neutral or basic aqueous medium to thereby directly produce a predominant amount of R(-)-mandelic acid or a derivative thereof, whereby the racemic R,S-mandelonitrile or a derivative thereof or benzaldehyde and a derivative thereof with hydrogen cyanide can be directly converted into R(-)-mandelic acid or a derivative thereof at a ratio of about 80 to 100% and at an optical purity of almost 100%_{ee} without performing any optical resolution.

FIELD OF THE INVENTION

This invention relates to R(-)-mandelic acid and a derivative thereof. More particularly, it relates to a process for producing R(-)-mandelic acid represented by the following general formula (III) or a derivative thereof. The process is carried out by using a microorganism belonging to the genus Rhodococcus which is capable of effecting nitrile asymmetric hydrolysis of R,S-mandelonitrile represented by the following general formula (I) or a derivative thereof. Compounds having a mandelic acid skeleton are highly valuable from an industrial viewpoint as starting materials for producing cephem-series antibiotics and a number of medicines and agricultural chemicals.

BACKGROUND OF THE INVENTION

Known methods for producing R(-)-mandelic acid and derivatives thereof include: racemic resolution of chemically synthesized R,S-mandelic acid (racemic modification) by (1) fractional crystallization as described in JP-A-58-177933 (the term "JP-A" as used herein means an "unexamined published Japanese patent application"); (2) chromatography as described in European Patent Publication No. 0 098 707A; and (3) conversion of the racemic modification into racemic esters followed by racemic resolution thereof by enzymatic asymmetric hydrolysis as described in K. Mori et al., Tetrahedron **36**, 91-96 (1980); and (4) chemical asymmetric synthesis with the use of a chiral reagent as described in D.A. Evans et al., J. Am. Chem. Soc., **107**, 4346-4348 (1985). Biological methods therefor include the asymmetric hydrolysis of the abovementioned esters formed by process (3) above and (5) microbiological asymmetric reduction of benzoylformic acid as described in JP-A-57-198096; (6) hydrolysis of R(-)-mandelonitrile and substituted derivatives thereof which are asymmetrically synthesized with the use of D-oxynitrilase as described in U.S. Patents 4,859,784 and 5,008,192; and (7) asymmetric hydrolysis of mandelonitrile, mandelamide and substituted derivatives thereof with the use of microorganisms belonging to the genus Alcaligenes, Pseudomonas, Rhodopseudomonas, Corynebacterium, Acinetobacter, Bacillus, Mycobacterium, Rhodococcus or Candida as described in European Patent Publication No. 0 348 901A.

However, each of the racemic resolution methods (1) to (3) requires a complicated process and a decrease in the yield occurs in each step. In method (4), using a chiral reagent as a catalyst, an expensive chiral reagent is required and a product of high optical purity is almost impossible to obtain.

The above-mentioned biological methods also have some disadvantages. Namely, in method (5) it is difficult to synthesize the substrate for the asymmetric reduction of benzoylformic acid. It is further difficult to maintain the NADH-regeneration system. The D-oxynitrilase method (6) is merely a basic finding that an optically active substance is obtained. The asymmetric hydrolysis method (7) requires a post treatment of another optically active substance remaining after the completion of the hydrolysis. In addition, the above-mentioned European Patent Publication No. 0 348 901A contains no particular example of the production of a R(-)-mandelic acid derivative from a substituted derivative of mandelonitrile. Therefore, it is unknown whether an R(-)-mandelic acid derivative of a high optical purity can be efficiently obtained or not.

As described above, these known methods suffer from various problems. Thus, none of these known methods is an industrially advantageous method for producing R(-)-mandelic acid and derivatives thereof.

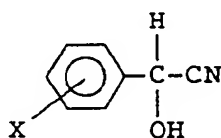
The present inventors conducted extensive investigations to establish a method for advantageously producing R(-)-mandelic acid on an industrial scale. As a result, the present inventors discovered that R,S-mandelonitrile could be easily racemized through dissociation equilibrium into benzaldehyde and hydrogen cyanide in an almost neutral or basic aqueous medium, and that R,S-mandelonitrile or benzaldehyde and hydrogen cyanide could be directly converted into R(-)-mandelic acid in conjunction with this racemization system together with a microorganism capable of asymmetric hydrolysis of mandelonitrile. Based on these findings, they disclosed a process for producing R(-)-mandelic acid by using a microorganism belonging to the genus Pseudomonas, Alcaligenes, Acinetobacter or Caseobacter as described in European Patent Publication No. 0 449 648A and another process for producing R(-)-mandelic acid with the use of a microorganism belonging to the genus Nocardia, Bacillus, Brevibacterium or Aureobacterium as described in European Patent Publication No. 0 449 648A. Subsequently, they further applied the above-mentioned findings to substituted mandelonitrile derivatives and, as a result, proposed a process for producing R(-)-mandelic acid derivatives with the use of a microorganism belonging to the genus Aureobacterium, Pseudomonas, Caseobacter, Alcaligenes, Acinetobacter, Brevibacterium or Nocardia as described in European Patent Publication No. 0 449 648A.

However, the R-stereoselectivities of the hydrogenases produced by these bacteria are not always satisfactory and thus further evaluation has been required for the stereoselectivities.

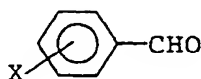
SUMMARY OF THE INVENTION

The present inventors have further attempted to find a microorganism possessing an excellent capability of asymmetrically hydrolyzing mandelonitrile and derivatives thereof. As a result, they have successfully discovered that an extremely high R-stereoselectivity can be achieved by using a microorganism belonging to the genus Rhodococcus, thus completing the present invention.

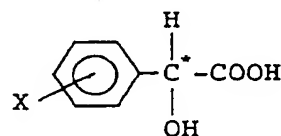
Accordingly, the present invention provides a process for producing a predominant amount of R(-)-mandelic acid represented by the following general formula (III) or a derivative thereof from R,S-mandelonitrile represented by the following general formula (I) or a derivative thereof or from benzaldehyde represented by the following general formula (II) or a derivative thereof, with hydrogen cyanide by treating a R,S-mandelonitrile of general formula (I) or a derivative thereof or a mixture of benzaldehyde of general formula (II) or a derivative thereof, with hydrogen cyanide using a microorganism, which may be treated, belonging to the genus Rhodococcus and capable of stereoselectively hydrolyzing the nitrile group in a R,S-mandelonitrile represented by the general formula (I) or a derivative thereof in a substantially neutral or basic aqueous medium:



(I)



(II)



(III)

wherein X, which is located at the o-, m- or p-position, is selected from the group consisting of a hydrogen atom, a halogen atom, a hydroxy group, an alkyl group having 1 to 3 carbon atoms, an alkoxy group having 1 to 3 carbon atoms, a thioalkyl group having 1 to 3 carbon atoms, an amino group, a nitro group, a mercapto group, a phenyl group and a phenoxy group.

DETAILED DESCRIPTION OF THE INVENTION

As described above, the present invention has been completed by further applying the above-mentioned findings of the present inventors (i.e., R,S-mandelonitrile, capable of being easily racemized through a dissociation equilibrium into benzaldehyde and hydrogen cyanide in a substantially neutral or basic aqueous medium and R,S-mandelonitrile, capable of being directly converted into R(-)-mandelic acid in conjunction with the above described racemization system together with a microorganism capable of R-stereoselective hydrolysis of mandelonitrile) to the R,S-mandelonitrile derivatives represented by the general formula (I) and by using a microorganism having an extremely high R-stereoselectivity as the above-mentioned microorganism.

The expression "predominant amount" as used herein means that the starting R,S-mandelonitrile represented by the general formula (I) and a derivative thereof or a mixture of benzaldehyde represented by the general formula (II) and a derivative thereof with hydrogen cyanide, can be directly converted into the target R(-)-mandelic acid represented by the general formula (III) and a derivative thereof at a ratio of "50% or above".

The microorganism to be used in the present invention is a microorganism belonging to the genus Rhodococcus. Rhodococcus sp. HT29-7 (FERM BP-3857) may be cited as a particular example thereof. This strain was isolated from soil by the present inventors for the first time and deposited with Fermentation Research Institute, Agency of Industrial Science and Technology, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki, Japan under the abovementioned accession number. The bacteriological properties of this strain are as follows.

Strain HT29-7:

Morphotype:	polymorphobacillus
Gram-staining:	+
Sporulation:	-
Motility:	-

	Colony color:	pink to orange
	Rod-coccus cycle:	+
5	Elongation of cell around colonies:	yes
	Formation of mycelia:	no
	Oxidase:	-
10	Catalase:	+
	Oxygen requirement:	aerobic
15	Diamino acid in cell wall:	meso-diaminopimelic acid
	Glycolyl test:	+ (glycolyl type)
	Sugar composition of cell wall:	
20	arabinose:	+
	galactose:	+
25	Quinone system:	MK-9 (H ₂)

Based on these bacteriological properties, this strain HT29-7 was identified as a bacterium belonging to the genus Rhodococcus according to Bergey's Manual of Systematic Bacteriology, 1986.

30 Examples of the compound represented by the above-mentioned general formula (I) include mandelonitrile, and chloromandelonitrile, bromomandelonitrile, fluoromandelonitrile, hydroxymandelonitrile, methylmandelonitrile, ethylmandelonitrile, isopropylmandelonitrile, methoxy-mandelonitrile, methylthiomandelonitrile, mercaptomandelonitrile, aminomandelonitrile, nitromandelonitrile, phenylmandelonitrile and phenoxymandelonitrile each having a substituent at the o-, m- or p-position.

35 The compound represented by the general formula (II) includes a benzaldehyde corresponding to the abovementioned compounds as well as derivatives thereof.

40 The microorganism to be used in the present invention may be incubated in a medium commonly employed in the art containing 0.5 to 20% carbon sources (for example, glycerol, glucose, saccharose) and 0.5 to 10% nitrogen sources (for example, casamino acids, meat extract, yeast extract), which can be utilized by the microorganism, and mineral salts (for example, magnesium chloride, calcium chloride, manganese sulfate, iron chloride, zinc sulfate) which are essential for the growth of the microorganism.

45 It is preferable to add nitriles (for example, cinnamyl nitrile, benzyl cyanide, isobutyronitrile, β -phenylpropionitrile, benzonitrile, 2-cyanopyridine, 3-cyanopyridine, 4-cyanopyridine, 1-cyclohexenyl acetonitrile, ϵ -caprolactam, ϵ -butyronitrile, o-aminobenzonitrile) or amides (for example, isobutylamide, phenylacetamide, 4-pyridylcarboxylic acid amide) to the medium at such a concentration so as not to considerably inhibit the growth of the microorganism during the early or medium stage of the incubation, since the enzymatic activity can be further elevated thereby.

The incubation may be carried out at a pH value of the medium of from 4 to 10 (preferably from 6 to 9) and at a temperature of from 5 to 50°C (preferably from 25 to 40°C) under aerobic conditions for approximately 1 to 14 days (preferably 3 to 7 days).

50 The hydrolysis may be carried out in the following manner. Cells or treated cells (for example, ground cells, crude or purified enzymes, immobilized cells, immobilized enzymes) of the microorganism, which has been incubated by the above-mentioned method, are suspended in an aqueous medium such as water or a buffer solution. Then, the obtained suspension is mixed with the R,S-mandelonitrile represented by the general formula (I) and a derivative thereof or a mixture of benzaldehyde represented by the general formula (II) or a derivative thereof with hydrogen cyanide. As described above, it is required in the present invention to maintain the reaction system at a substantially neutral or basic state throughout the reaction in order to racemize the mandelonitrile and a derivative thereof. Therefore, the pH value of the reaction system is adjusted to from 6 to 11 (preferably from 7 to 9).

In the reaction mixture, the content of the mandelo-nitrile and a derivative thereof may range from 0.1 to 10% by weight (preferably from 0.2 to 5.0% by weight), the content of the benzaldehyde and a derivative thereof may range from 0.1 to 10% by weight (preferably from 0.2 to 5.0% by weight) and the content of the hydrogen cyanide may range from 0.1 to 2.0% by weight (preferably from 0.1 to 0.5% by weight). Based on the substrates, the micro-organism may be used at a ratio of from 0.01 to 5.0% by dry weight (preferably from 0.1 to 2% by dry weight). The reaction temperature may range from 0 to 50°C, preferably from 10 to 30°C. The reaction may be performed for 0.1 to 100 hours (preferably for 5 to 30 hours).

The R(-)-mandelic acid thus obtained and a derivative thereof are then treated as follows. The cells are removed from the reaction mixture by a known procedure such as centrifugation and then granular components, proteins and polysaccharide components are further removed, if required, by, for example, ultrafiltration. Then, the residue is optionally treated with activated carbon and concentrated under reduced pressure, or extracted with an organic solvent under acidic conditions. After repeated recrystallization from, for example, benzene, the target product is obtained as crystals of a high purity.

According to the present invention, R,S-mandelonitrile of racemic modification and a derivative thereof or benzaldehyde and a derivative thereof with hydrogen cyanide can be directly converted into R(-)-mandelic acid and a derivative thereof at a ratio of about 80 to 100% and at an optical purity of almost 100%_{ee} without performing any optical resolution. Thus, the process for producing R(-)-mandelic acid and a derivative thereof of the present invention is highly superior to known methods in terms of operation and cost.

The following Examples are given to further illustrate the present invention in greater detail, and not for the purpose of limiting thereto.

EXAMPLE 1

(1) Incubation of Microorganism and Preparation of Cell Suspension:

Rhodococcus sp. HT29-7 was incubated in the following medium A at 30°C for 72 hours and the cells thus obtained were further incubated in the medium B at 30°C for 96 hours.

i) Media

(Medium A)

Glycerol	20 g
Yeast extract	6 g
Monopotassium phosphate	6.8 g
Dipotassium phosphate	7.1 g
Sodium sulfate	2.8 g
Magnesium chloride	0.4 g
Calcium chloride	0.04 g
Manganese sulfate	0.03 g
Iron chloride	0.006 g
Zinc sulfate	0.003 g
Distilled water	1000 ml
pH	7.5

(Medium B)

0.02% (weight/volume) of 1-cyclohexenyl acetonitrile was further added to the above Medium A.

The cells were separated from the obtained incubation broth and washed with a 50 mM phosphate buffer solution (pH 8.0). Next, the cells were suspended in 100 ml of this same phosphate buffer solution to form a cell suspension ($OD_{630} = 28.3$, 1.13% by dry weight).

(2) Asymmetric hydrolysis of mandelonitrile:

To the cell suspension obtained above, mandelonitrile was continuously added with the use of a micro-test tube pump at a flow rate of 5.35 mmol/hr and the reaction was carried out at 30°C. After adding mandelonitrile for one hour, R(-)-mandelic acid and ammonia were formed substantially quantitatively based on the consumed mandelonitrile.

The reaction was carried out for 4 hours total under the above-mentioned conditions. When analyzed by liquid chromatography with the use of an ODS column, 3.4% (weight/volume) of ammonium R(-)-mandelate was accumulated from 2.8 g of the starting mandelonitrile 4 hours after the initiation of the reaction (conversion yield: 94.1%). After the completion of the reaction, the cells were removed from the reaction mixture, the thus obtained reaction mixture was treated with an acid so as to give a pH value of 2. The aqueous phase was then extracted with ethyl acetate, thereby forming mandelic acid. The aqueous solution of mandelic acid thus obtained was dried over sodium sulfate anhydride. The organic solvent was then distilled off, thereby forming crude crystals. The crystals were further recrystallized from a mixed solvent of benzene/ethyl acetate (7 : 1 by volume) to thereby form 2.8 g of crystals in the form of a white powder. These crystals and standard R(-)-mandelic acid were each formulated into an aqueous solution of 1.0% (weight/volume) using distilled water. Next, the specific rotation of each aqueous solution was measured with a rotational meter and the optical purity thereof was determined by high performance liquid chromatography using an optical resolution column (MCI GEL CRS10W). The analytical data thus obtained is shown in Table 1 below.

Table 1

<u>Sample</u>	<u>Specific Rotation</u> $[\alpha]_D^{20}$	<u>Optical Purity</u> (%)
Example 1	-150	100
Standard	-153	100

EXAMPLE 2

(1) Incubation of Microorganism and Preparation of Cell Suspension:

Rhodococcus sp. HT29-7 was incubated under the same conditions and a cell suspension was prepared (OD₆₃₀ = 12, 0.48% by dry weight) with 50 mM phosphate buffer solution at pH 8.0, as those described in Example 1.

(2) Asymmetric Hydrolysis of Various Substrates:

Various mandelonitrile derivatives or hydrogen cyanide and benzaldehyde derivatives as listed in Table 2 were added to the 10 ml each of above-mentioned cell suspension each at a concentration specified in Table 2 and then the reaction was carried out at 30°C for 17 hours while shaking.

After the completion of the reaction, the cells were removed by centrifugation from the reaction mixture and the reaction yield and the optical purity of the obtained product were determined by high performance liquid chromatography with the use of both ODS and optical resolution column (MCI GEL CRS10W) by the same method as the one described in Example 1. The results are shown in Table 2 below.

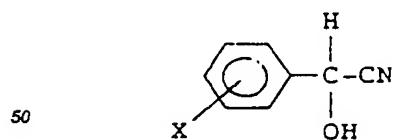
Table 2

		<u>R(-)-Mandelic Acid/Derivative</u>				
5	<u>Substrate</u>	<u>Amount</u>	<u>X</u>	<u>Formed</u>	<u>Yield</u>	<u>Optical</u>
		(mM)		(mM)	(%)	Purity
						(% _{ee})
	Benzaldehyde	10	H-	9.8	98	100
10	Hydrogen Cyanide	11				
	2-Chloromandelonitrile	7	2Cl-	6.8	97	100
	3-Chloromandelonitrile	6	3Cl-	4.6	77	100
15	4-Chloromandelonitrile	7	4Cl-	4.5	64	100
	4-Hydroxybenzaldehyde	4	4HO-	3.5	88	100
20	Hydrogen Cyanide	4				
	4-Methylbenzaldehyde	4	4CH ₃ -	3.7	93	100
	Hydrogen Cyanide	5				
25	4-Methoxybenzaldehyde	3	4CH ₃ O-	2.5	83	100
	Prussic acid	3				

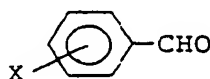
30 While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

35 Claims

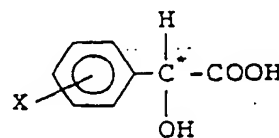
1. A process for producing a predominant amount of R(-)-mandelic acid represented by the following general formula (III) or a derivative thereof directly from R,S-mandelonitrile represented by the following general formula (I) or a derivative thereof or from benzaldehyde represented by the following general formula (II) or a derivative thereof with hydrogen cyanide, by treating (A) the R,S-mandelonitrile of general formula (I) or a derivative thereof or (B) a mixture of benzaldehyde of general formula (II) or a derivative thereof with hydrogen cyanide, using a microorganism belonging to the genus *Rhodococcus* and capable of stereoselectively hydrolyzing the nitrile group of the R,S-mandelonitrile represented by the general formula (I) or a derivative thereof in a substantially neutral or basic aqueous medium:



(I)



(II)



(III)

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wherein X, which is located at the o-, m- or p-position, is selected from the group consisting of a hydrogen atom, a halogen atom, a hydroxy group, an alkyl group having 1 to 3 carbon atoms, an alkoxy group having 1 to 3 carbon atoms,

a thioalkyl group having 1 to 3 carbon atoms, an amino group, a nitro group, a mercapto group, a phenyl group and a phenoxy group.

2. The process for producing R(-)-mandelic acid as claimed in claim 1, wherein R(-)-mandelic acid or a derivative thereof are formed in a ratio of at least 50%. 5
3. The process for producing R(-)-mandelic acid as claimed in claim 1, wherein R(-)-mandelic acid or a derivative thereof are formed in a ratio of 80 to 100%.
4. The process for producing R(-)-mandelic acid as claimed in claim 1, 2 or 3 wherein the R,S-mandelonitrile is selected from the group consisting of mandelonitrile, chloromandelonitrile, bromomandelonitrile, fluoromandelonitrile, hydroxymandelonitrile, methylmandelonitrile, ethylmandelonitrile, isopropylmandelonitrile, methoxymandelonitrile, methylthiomandelonitrile, mercaptomandelonitrile, aminomandelonitrile, nitromandelonitrile, phenylmandelonitrile and phenoxymandelonitrile each having a substituent at the o-, m- or p-position. 10 15
5. The process for producing R(-)-mandelic acid as claimed in claim 1, 2, 3 or 4, wherein the pH value of said aqueous medium is from 6 to 11.
6. The process as claimed in any preceding claim, wherein the microorganism has the R-stereoselective properties of Rhodococcus sp. HT29-7 (FERM BP-3857). 20

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European Patent
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EUROPEAN SEARCH REPORT

Application Number

DOCUMENTS CONSIDERED TO BE RELEVANT			EP 92305814.3
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
D,X	<u>EP - A - 0 348 901</u> (ASAHI KASEI KOGYO KABUSHIKI KAISHA) * Claim 1; page 10 * ---	1	C 12 P 7/42 C 12 P 41/00
D,P, X	<u>EP - A - 0 449 648</u> (NITTO CHEMICAL INDUSTRY) * Claim 1 * ---	1	
D,A	PATENT ABSTRACTS OF JAPAN, unexamined applications, C field, vol. 7, no. 46, February 23, 1983 THE PATENT OFFICE JAPANESE GOVERNMENT page 62 C 153 * Kokai-no. 57-198 096 (KANEGAFUCHI KAGAKU KOGYO K.K.) * -----	1	
			TECHNICAL FIELDS SEARCHED (Int. Cl.5)
			C 12 P
The present search report has been drawn up for all claims			
Place of search VIENNA		Date of completion of the search 01-09-1992	Examiner WOLF
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			

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